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13. ABSTRACT (Maximum 200 Words)

Sphingosine kinase 1 (SphK1) and its product sphingosine 1-phosphate have been shown to promote cell growth and inhibit apoptosis of tumor cells (reviewed in [1]). SphK1 has been shown to be responsible for radioresistance of certain prostate cancer cells [2]. To better understand SphK1 regulation, we undertook a two-hybrid screen for SphK1-interacting proteins. In the first report period, we focused on one of these interactors, aminoacylase 1. This work will not be discussed as it has been accepted for publication (appendix A). In this report period we studied a second interacting protein, filamin A. We show that SphK1 physically interacts with both the fragment of filamin found in the two-hybrid screen and full length. Though both C-terminal and full length proteins reduce SphK1 activity measured *in vitro*, the C-terminal fragment inhibits while the full length potentiates the effects of SphK1 on TNF-α signaling and motility. We further demonstrate that filamin is required for ligand-induced motility as well as activation of SphK1. Moreover, siRNA against SphK1 suggests the SphK1-filamin interaction is required for motility, indicating possible anti-metastasis drug targets.

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Introduction

Sphingolipids are ubiquitous constituents of eukaryotic membranes characterized by the presence of an acylated sphingoid base, ceramide (Cer). Cer and its further metabolites sphingosine (Sph) and Sph-1-phosphate (S1P) are now recognized as potent bioactive molecules. In many cell types, increased Cer and Sph levels lead to cell growth arrest and apoptosis (reviewed in [1, 3, 4]). Conversely, S1P promotes cell growth and inhibits apoptosis (reviewed in [1, 5, 6]). Cells contain signal-regulated enzymes that can interconvert Cer, Sph, and S1P. Thus, conversion of Cer and Sph to S1P simultaneously removes pro-apoptotic signals and creates a survival signal, and vice versa. This led to the proposal of a "sphingolipid rheostat" as a factor determining cell fate [7]. According to this hypothesis, it is not the absolute levels but the relative amounts of these antagonistic metabolites that determines cell fate. In agreement, it has been shown that increased S1P protects against Cer-induced apoptosis, and depletion of S1P enhances Cer-induced apoptosis [7-10].

There are a number of agonists, especially growth and survival factors, that have been reported to increase SphK activity, including ligands for G-protein coupled receptors [11-13] and growth factor receptors [8, 14, 15]. Activation of SphK is required for at least some of the signaling effects observed. Requirement for SphK activation was typically based on the ability of inhibitors of SphK, including dominant negative SphK1 [16], to block agonist-induced effects and/or the ability of exogenously added S1P or a precursor to bypass the agonist. While many early studies suggested a role for S1P as an intracellular second messenger, it was later demonstrated that S1P is also a ligand for a family of G-protein coupled receptors (reviewed in [17]). Complicating matters, there is growing evidence that agonist-induced SphK activation leads to S1P secretion [18, 19] and autocrine and/or paracrine signaling to the cell surface S1P receptors [20, 21].

SphK1 and S1P have been linked to growth, metastasis, and radio- and chemotherapy resistance of tumors, including prostate tumors (reviewed in [1]). For example, it was shown that in radiation sensitive prostate cancer cells, γ-irradiation reduces SphK1 activity, leading to increased Cer and Sph levels and subsequent apoptosis. However, radiation-resistant prostate cancer cells showed no change in SphK activity or Cer levels. Furthermore, inhibitors of SphK sensitized these cells to γ-irradiation, demonstrating a role for SphK in prostate tumor radiation resistance [2].

In order to better understand the regulation and activation of SphK1, we had performed a two-hybrid screen for protein interactors of SphK1. In the initial proposal, we set out to characterize several of these interactors and their potential physiological influence on SphK1. In our first Annual Report, we discussed our results with one of these interactors, aminoacylase 1. That work was submitted and accepted for publication by FEBS Letters and is included as appendix A. Here we discuss the work with a second interacting protein, filamin A.

Updated Results

A C-terminal fragment of filamin A was pulled out of a kidney cDNA library with a two-hybrid screen as an interactor with mouse SphK1. Filamin, also know as filamin 1 and ABP280, is a 280 kDa protein that acts as a dimer. The N-terminus of the protein has an actin binding domain, while the central and C-terminal portions of the protein have coiled-coiled domains responsible for dimerization and protein-protein interactions (reviewed in [22]). While first thought of a structural protein of the cytoskeleton, filamin is emerging as an important scaffolding molecule involved in cell signaling and endocytosis, having been found to interact with TRAF2 [23], PAK [24], and integrins [25], among others. Intriguingly, filamin has also been shown to physically interact with and to be required for the proper localization of PSMA, a protein highly expressed in prostate cancer but not normal tissue [26]. As a first step in analyzing SphK1-filamin interactions, we confirmed the two-hybrid data by showing that SphK1 and the C-terminal fragment could interact when co-expressed in mammalian cells (**Task 1c**; figure 1, upper panel). It has been reported that SphK1 physically interacts with TRAF2 [27], and that this interaction is required for TRAF2-mediated signaling in response to TNF-α. Moreover, TRAF2 has been shown to interact with filamin [23]. Therefore, we re-probed our blot with antibodies to TRAF2. As expected, SphK1 co-purified TRAF2. Interestingly, the amount of TRAF2 that co-purifies with SphK1 is independent of filamin expression (figure 1, lower panel, lanes 1 and 4). This suggests that SphK1 binds filamin and TRAF2 at independent sites, and that the three can be co-purified as a complex. There are good antibodies commercially available against endogenous filamin, and we have developed in our laboratory a polyclonal antibody which recognizes endogenous SphK1 (**Task 1a**). Using the antibody to SphK1, we were able to co-immunoprecipitate filamin from HEK 293 cells, (**Task 1d**; figure 2), demonstrating that the interaction between the two proteins is not an artifact of over-expression, and that the interaction occurs not just between mouse proteins (used in the original screen and in over-expression) but also the human proteins.

These results suggest that SphK1 and filamin physically interact *in vivo*. The next question was what are the physiological ramifications of this interaction. SphK1 assays were performed on TNF-α-stimulated HEK 293 cells expressing either vector or SphK1 and either vector or C-terminal filamin (**Task 1e**, figure 3). Intriguingly, the C-terminal fragment of filamin inhibited stimulated but not basal SphK1 activity, in both vector and SphK1 expressing cells, suggesting that it acts as a dominant negative inhibitor of SphK1 in response to TNF-α signaling. This is likely due at least in part to the fact that the C-terminal filamin construct lacks the actin binding domain, and thus would not be able to translocate SphK1 to the cytoskeleton. To determine the effect of inhibiting SphK1 activity, we examined a downstream effect of TNF-α stimulation by examining p38, a kinase phosphorylated in response to TNF-α. Again, cells expressing either vector or SphK1 and either vector or C-terminal filamin were stimulated with TNF-α and lysates blotted with phospho-p38 specific antibodies (figure 4, upper panel). Expression of SphK1 enhanced the phosphorylation of p38, and C-terminal filamin reduced this effect, again suggesting that it is acting as a dominant negative inhibitor of at least some aspects of SphK1 signaling. Similar results were obtained when phosphorylation of the related kinases, p44/p42-ERK (MAPK), were examined (figure 4, lower panel). Because TRAF2 shifts TNF-α to promote cell growth, we plan to extend these results by examining the effect of SphK1-filamin interactions in promoting cell growth and inhibiting apoptosis in response to TNF-α.

Because the two-hybrid screen yielded only a C-terminal portion of filamin, we obtained a full length clone from the lab of Dr. T.P. Stossel (**Task 1d**; data not shown). We also received two cell lines: M2, melanoma cells which express little or no filamin, and A7 cells, M2 cells engineered to stably express filamin [28]. These cells have been used to examine the role of filamin in cell motility, a major contributor to metastasis [24]. This is intriguing because much work has demonstrated that S1P, the product of SphK1, acts through cell surface G-protein coupled receptors to either promote or inhibit cell motility, depending on the receptor (reviewed in [29]). Moreover, we have demonstrated that SphK1 translocates from the cytosol to lamellapodia in response to chemoattractants [21], consistent with S1P being released and acting in an autocrine and/or paracrine manner [20, 30]. We hypothesize that SphK1 translocates to the leading edge of the cell by binding to filamin, which is also known to localize to the leading edge upon stimulation [24]. There, SphK1 makes S1P, which is secreted and activates pro-migratory S1P receptors. This "inside-out" signaling of ligand to SphK1 to S1Pr has been observed in several systems, including PDGF [20] and Fcε receptor cross-linking [30].

As a first step, we determined by real time-PCR that M2 and A7 cells express SphK1 and receptors S1P1, 2, 3, and 5 but not S1P4 (data not shown). Heregulin (Hrg) stimulates migration in the filamin-containing A7 but not filamin-negative M2 cells [24]. When A7 cells were stimulated with (Hrg), SphK1 activity increased, while no change in activity was observed in M2 cells (figure 5, open bars). This is consistent with our hypothesis that filamin is required for activation of SphK1. Because we planned to use siRNA directed against SphK1 in these cells, as a control we tested this siRNA to ensure that it reduced SphK1 activity, which it does (figure 5, shaded bars). As a further control, we tested whether or not our antibody directed against SphK1 detected the protein in these cells (figure 5, lower panel). Indeed, our antibody recognizes a single band near the expected molecular weight. Additionally, this band is undetectable when cells are treated with siRNA directed against SphK1. Immunocytochemistry and cell fractionation experiments are ongoing to determine if SphK1 and filamin co-localize to the leading edge of migratory cells, and whether C-terminal filamin disrupts this localization.

We then performed modified Boyden chamber migration assays [20] to assess the role of SphK1 in motility in the M2 and A7 cells. In no case did we observe ligand-induce migration in the filamin-negative M2 cells (data not shown). As expected, Hrg induced migration in A7 cells (figure 6). Interestingly, when A7 cells were transfected with SphK1, no increase over vector stimulation was observed, suggesting that there is sufficient endogenous SphK1 to give maximal migration. However, when SphK1 levels were reduced with SphK1-specific siRNA or when C-terminal filamin was expressed, basal and Hrg-stimulated migration was reduced. Similar results were observed in HEK cells treated with EGF (data not shown). If the SphK1 recruitment is necessary for S1P production and secretion to activate S1P receptors, then A7 cells would be expected to migrate towards S1P. Indeed, this is exactly what was observed: S1P stimulated migration of A7 cells that was comparable to Hrg (figure 7). The decreasing response to higher concentrations of S1P has been observed many times (e.g. [20]), and may be due to stimulation of lower affinity S1P receptors which known inhibit cell motility (i.e. S1P2). To confirm the "inside-out" signaling, we plan to measure S1P secretion upon Hrg stimulation, and to use siRNA to determine which S1P receptor is involved, likely S1P1, 3 or both.

Key Research Results

- SphK1 physically interacts with both the C-terminus of filamin as well as full length.
- C-terminal fragment of filamin may act as a dominant negative inhibitor of SphK1 activity.
- C-terminal fragment of filamin may act as a dominant negative regulator of TNF-α.
- SphK1 likely forms a signaling complex with filamin and TRAF2 to mediate the pro-growth signaling of TNF-α.
- M2 and A7 cell data suggest SphK1-filamin interaction is required for cell migration in response to heregulin.
- Migration of A7 cells to S1P alone suggests "inside-out" signaling.

Reportable Outcomes

Published Paper: Michael Maceyka, Victor Nava, Sheldon Milstien, and Sarah Spiegel. *Sphingosine kinase 1 interacts with aminoacylase 1.* FEBS Lett, 2004 (In Press).

Conclusion

The data accumulated in the first reporting period strongly suggests that SphK1 physically and physiologically interacts with the C-terminal third of Acy1, work which is in press (Appendix A). In the second reporting period, we have focused our efforts on a second SphK1 interacting protein, filamin a. We have found that the interaction of SphK1 with filamin is required for certain aspects of TNF-α signaling. This is important because TNF-α can promote cell growth or cell death, depending on the accessory molecules with interact with the activated receptor. The TRAF2-mediated signaling normally promotes cell growth and inhibits apoptosis, in part through its interaction with SphK1. Thus, the interaction between SphK1, filamin, and TRAF2 may provide useful targets for intervention in cancer therapy. Moreover, the interaction between filamin and SphK1 is involved in the regulation of motility, a necessity for metastasis. Intriguingly, a recent report demonstrates that PSMA, a protein up-regulated in prostate tumors but not normal tissue, interacts with filamin [26]. It may be that these three proteins, filamin, SphK1, and PSMA work in concert to promote prostate tumors. Thus, increased understanding of the interaction of these proteins may provide novel targets for disrupting the spread of prostatic tumors.

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Figure 1: SphK1 interacts with the C-terminus of filamin and with TRAF2. Vecotr (V) or V5-6xHis-tagged SphK1 (K1) was expressed in the absence and presence (K1H) of HA-tagged CT-filamin (HA fil) in HEK cells. The lysates were incubated with Ni-agarose to purify SphK1 and blotted for the presence of CT filamin. V, vector transfected cells. The same blot was also probed with antibodies to TRAF2. lower panel.

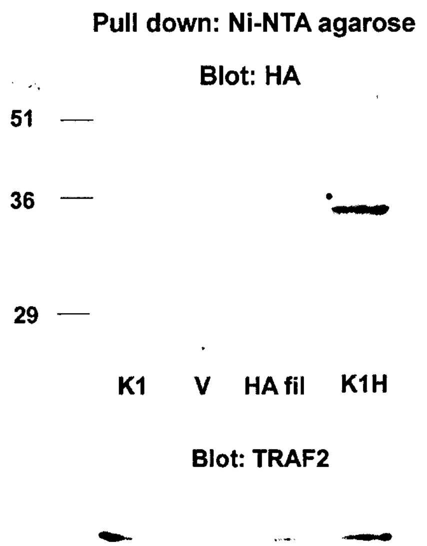


Figure 3: Expression of C-terminal filamin inhibits TNF-α stimulated SphK1 activity. HEK cells were transfected with the indicated constructs and stimulated without or with TNF for 10 min. Lysates were prepared and assayed for SphK1 activity. Representative of 3 experiments.

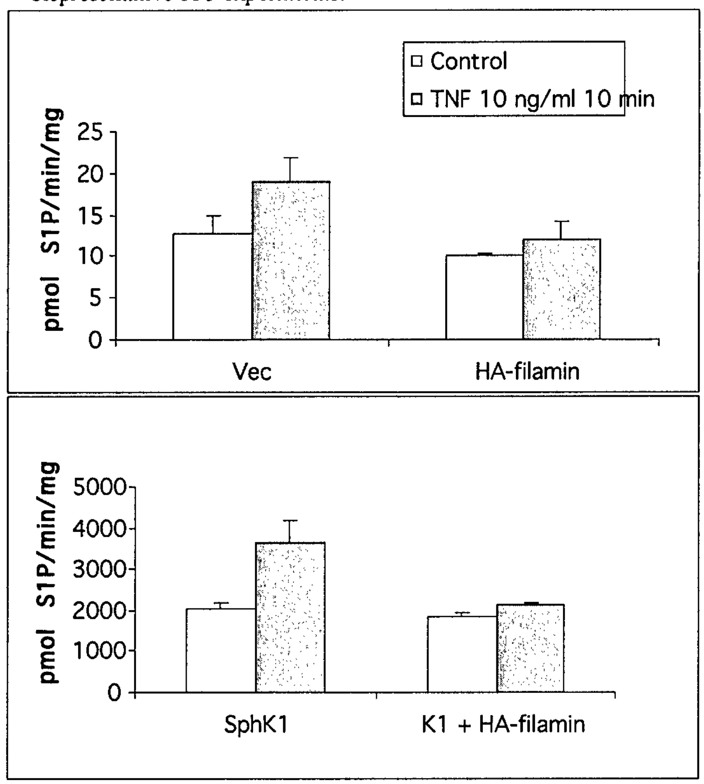


Figure 2: Endogenous SphK1 interacts with endogenous filamin. A lysate was prepared from naïve HEK cells, and equal portions were incubated with pre-immune (mock) or post-immune serum (hSK1) specific for SphK1. Immune complexes were precipitated, washed and blotted for endogenous filamin. Representative of 3 experiments

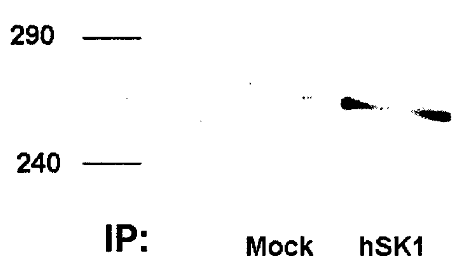


Figure 4: Expression of C-terminal filamin inhibits TNF-α stimulated signaling. HEK cells were transfected with the indicated constructs and stimulated without or with 10 ng/ml TNF for 10 min. Lysates were blotted for phospho-p38 (upper panel) and phospho-MAPK (lower panel). Loading control showed equal loading. Representative of 3 experiments.

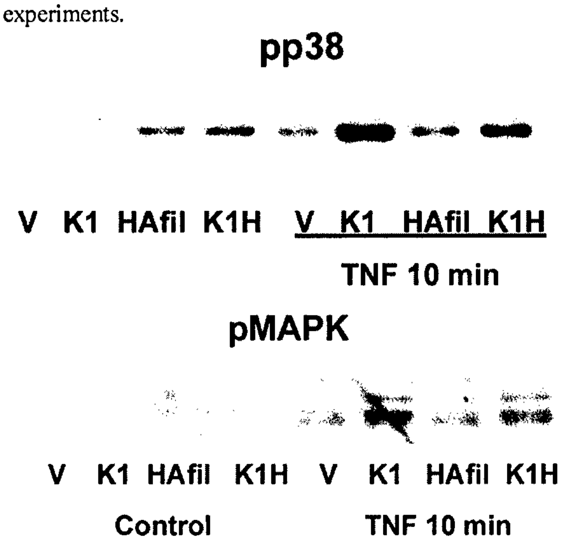


Figure 5: Hrg stimulates SphK1 in filamin expressing A7 but not filamin negative M2 cells, and siRNA directed against SphK1 reduces SphK1 activity. M2 and A7 were transfected with either control siRNA or siRNA directed against SphK1. Cells were then stimulated with Hrg for the indicated times, lysates prepared, and SphK1 activity measured. Representative of 3 experiments. Lower panel indicated western blot using anti-SphK1 antibodies. Left lane is molecular weight markers (in kDa), * indicates SphK1

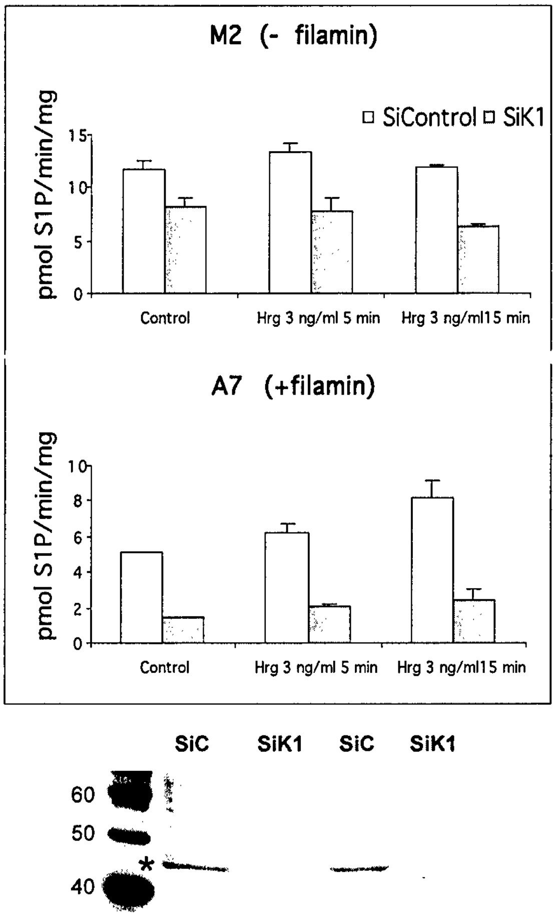


Figure 6: Hrg-stimulated migration in filamin expressing A7 cells is reduced by siRNA directed against SphK1 and by C-terminal filamin. A7 were transfected with the indicated constructs. Cells were then placed in a Boyden chamber and stimulated to migrate through a filter without (open bars) or with (shaded bars) Hrg for for 4 h. Representative of 2 experiments.

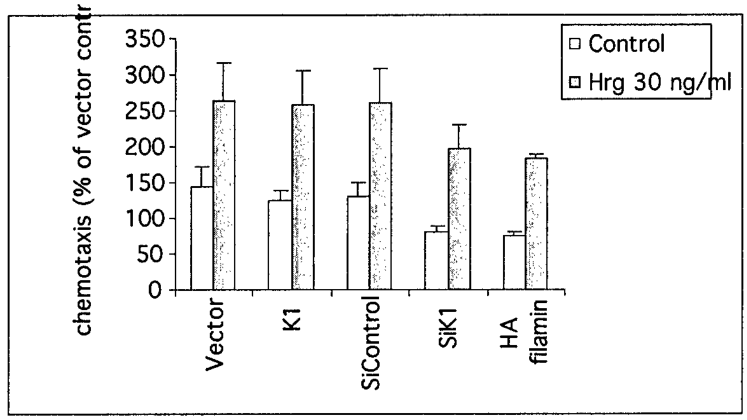
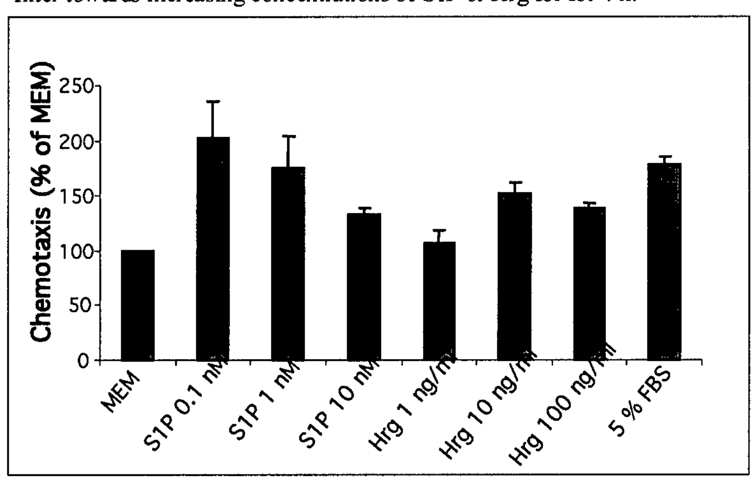


Figure 7: S1P-stimulated migration in filamin expressing A7 cells is reduced comparable to Hrg. A migration assay was performed with A7 cells. Cells were placed in a Boyden chamber and stimulated to migrate through a filter towards increasing concentrations of S1P or Hrg for for 4 h.



Aminoacylase 1 is a sphingosine kinase 1-interacting protein

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Abstract **Sphingosine kinase type 1 (SphK1) and its product sphingosine-1-phosphate have been shown to promote cell growth and inhibit apoptosis of tumor cells. In an effort to further understand the regulation of SphK1, we used a yeast two-hybrid screen to find SphK1-interacting proteins. One of these was identified as aminoacylase 1 (Acy1), a metalloenzyme that removes amide-linked acyl groups from amino acids and may play a role in regulating responses to oxidative stress. Both the C-terminal fragment found in the two-hybrid screen and full-length Acy1 co-immunoprecipitate with SphK1. Though both C-terminal and full-length proteins slightly reduce SphK1 activity measured in vitro, the C-terminal fragment inhibits while full-length Acy1 potentiates the effects of SphK1 on proliferation and apoptosis. Interestingly, Acy1 induces redistribution of SphK1 as observed by immunocytochemistry and subcellular fractionation. Collectively, our data suggest that acyl physically interacts with SphK1 and may influence its physiological functions.**

Keywords: Aminoacylase 1; Sphingosine kinase; Sphingosine; Sphingosine-1-phosphate; Yeast two-hybrid

1. Introduction

Sphingolipids are ubiquitous constituents of eukaryotic membranes whose backbones consist of an acylated sphingoid base, ceramide. Ceramide and its further metabolites, sphingosine and sphingosine-1-phosphate (S1P), are now recognized as potent signaling molecules. In many cell types, increased ceramide and sphingosine levels lead to cell growth arrest and apoptosis [1,2]. Conversely, S1P promotes cell growth and inhibits apoptosis [3–5]. Cells contain enzymes that can rapidly interconvert ceramide, sphingosine, and S1P. Thus, conversion of ceramide and sphingosine to S1P simultaneously removes pro-apoptotic signals and creates a survival signal, and vice versa [6–9]. While many early studies suggested a role for S1P as an intracellular second messenger, it was later convincingly demonstrated that S1P is also a ligand for a family of G protein-coupled receptors [5,10]. Complicating matters, there is growing evidence that agonist-induced sphingosine kinase type (SphK) activation leads to S1P secretion [11,12] and autocrine and/or paracrine signaling through cell surface S1P receptors [13–15].

Recently, progress has been made in elucidating the molecular mechanisms of activation of SphK type 1 (SphK1). It has been shown that PKC can phosphorylate SphK1, both activating SphK1 and inducing its translocation to the plasma membrane [12]. More recently, it has been demonstrated that activation and translocation of SphK1 from the cytosol to the plasma membrane results directly from phosphorylation at Ser225 by ERK1/2 [16]. SphK1 interacts with TRAF2, an interaction that is required for suppression of apoptosis by TNF-α [17]. Several other SphK1-interacting proteins have also recently been identified, including PECAM-1 [18], RPK118 [19], and AKAP-related protein SKIP1 [20], which are involved in the translocation of SphK1 to the plasma membrane, endosomes, and signaling complexes, respectively.

In a yeast two-hybrid search for additional SphK1-interacting proteins, we cloned aminoacylase 1 (Acy1) and showed that it interacted with SphK1 and affected its activity and biological functions.

2. Materials and methods

2.1. Cell culture and transfection

Cos7, HEK 293, and NIH 3T3 cells were obtained from ATCC. Cells were cultured in DMEM supplemented with 10% fetal bovine (Cos7, HEK) or 10% calf serum (NIH) and maintained at 37 °C in a humidified environment in 5% CO_2. All culture reagents were from BioFluids. HEK 293 cells, plated on poly-D-lysine, and NIH 3T3 cells were transfected using Lipofectamine Plus and Cos7 cells with Lipofectamine 2000 (Invitrogen).

2.2. Two-hybrid screen and cloning

The two-hybrid screen was carried out using the MatchMaker II Kit from Clontech as described [20] with mouse SphK1a as bait against a mouse kidney cDNA library (Clontech). A clone of the C-terminal portion of Acy1 (CT-Acy1) was obtained from this screen that passed all tests as a valid two-hybrid interactor. The CT-Acy1 was removed from the library vector using *Eco*RI and *Bam*H1 and cloned into pcDNA3-HA (N-terminal tag). Full-length Acy1 was cloned by PCR from a mouse kidney library using the V5-His-Topo Cloning Kit (Invitrogen).

2.3. Sphingosine kinase assay

SphK1 activity was measured essentially as described [21] with sphingosine solubilized in Triton X-100 (0.25% final concentration).

2.4. GST pulldown and immunoprecipitation

The CT-Acy1 was transcribed and translated in vitro with the TnT Kit (Promega) in the presence of [^{3}H]leucine. The translation mix was

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Abbreviations: Acy1, aminoacylase 1; S1P, sphingosine-1-phosphate; SphK1, sphingosine kinase type 1

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incubated with either GST or GST-SphK1 as described [20], then affinity-purified using glutathione–Sepharose beads (Pierce), and washed three times with SphK assay buffer containing 1% Triton X-100. The pellet was resuspended in sample buffer and proteins resolved by SDS–PAGE. Gels were dried and exposed to film. For immunoprecipitation, HEK 293 transfectants were lysed and 800 μg lysate incubated with anti-myc antibodies for 24 h at 4 °C. Anti-myc immunocomplexes were precipitated with protein A/G Sepharose (Santa Cruz) and washed three times with SphK assay buffer containing 1% Triton X-100. The pellets were resuspended in sample buffer, proteins resolved by SDS–PAGE, and immunoblotted with anti-HA (CT-Acy1) or anti-V5 (Acy1).

2.5. Apoptosis and MTT assays

48 h after transfection, NIH 3T3 cells were serum-starved for 24 h to induce apoptosis. Cells were fixed with 4% paraformaldehyde in 4% sucrose–PBS and stained with 8 μg/ml Hoechst. Apoptotic nuclei were scored essentially as described [20]. Cell viability was assessed by the MTT dye reduction assay (Roche).

2.6. Fractionation and immunofluorescence

Cells were plated on 10-cm dishes. 48 h after transfection, cells were washed and harvested in SphK buffer. Cells were lysed by freeze–thaw and then centrifuged at 100 000 × *g*. Supernatants were removed (cytosol) and pellets washed with SphK buffer. Pellets were then resuspended in SphK buffer containing 1% Triton X-100 and solubilized on ice for 1 h. Solubilized pelletS were centrifuged at 100 000 × *g* for 30 min and supernatants (Triton soluble, TS) and pellets (Triton insoluble, TI) were then separated. TI pellets were resuspended in SphK buffer plus 1% Triton X-100. Western blotting was used to determine protein expression with either anti-myc (9E10; Santa Cruz), anti-HA (3F10; Roche), or anti-V5 (monoclonal from Invitrogen or rabbit polyclonal from Sigma–Aldrich) as primary antibodies followed by HRP-conjugated secondary antibodies (1:10 000, Jackson ImmunoResearch Laboratories). Immunocomplexes were visualized by enhanced chemiluminescence (Pierce) as described previously [22].

For immunofluorescence, cells were plated on #1 coverslips, transfected, and after 48 h, fixed in 3.7% formalin and stained essentially as described [20]. Briefly, after washing with PBS containing 10 mM glycine, cells were permeabilized for 3 min with 0.5% Triton X-100 in PBS–glycine, washed again, and incubated for 20 min at room temperature with mouse monoclonal anti-myc (2 μg/ml) for detection of SphK1 and rabbit anti-V5 (4 μg/ml) for Acy1. After washing, cells were incubated for 20 min with Texas Red-conjugated anti-mouse and FITC-conjugated anti-rabbit secondary antibodies (1 μg/ml each; Jackson ImmunoResearch). Coverslips were then mounted with glycerol containing 10 mM *n*-propyl gallate and images collected with a Nikon TE-200 fluorescence microscope.

3. Results and discussion

3.1. Acy1 is a SphK1-interacting protein

To search for proteins that interact with SphK1 and regulate its activity or translocation to the plasma membrane, a two-hybrid screen was carried out using mouse SphK1 a fused to the DNA binding domain of GAL4 as bait. The prey consisted of a mouse kidney cDNA library (Clontech) fused to the transcriptional activation domain of GAL4. Interaction between SphK1 and a library protein brings together the two domains necessary for transcription of reporter genes. The MatchMaker II system mitigates against false positives by having three different promoter–reporter gene constructs, with differing affinities for the GAL4 DNA-binding domain. This reduces the chances that the prey construct activates on its own by binding regions around the GAL4 DNA binding site or to specific TATA boxes and allows for control of stringency. Using the most stringent interaction test, a clone of the CT-Acy1, starting at amino acid 232 of the full-length protein (Fig. 1A), was obtained. Acy1 has been characterized as a

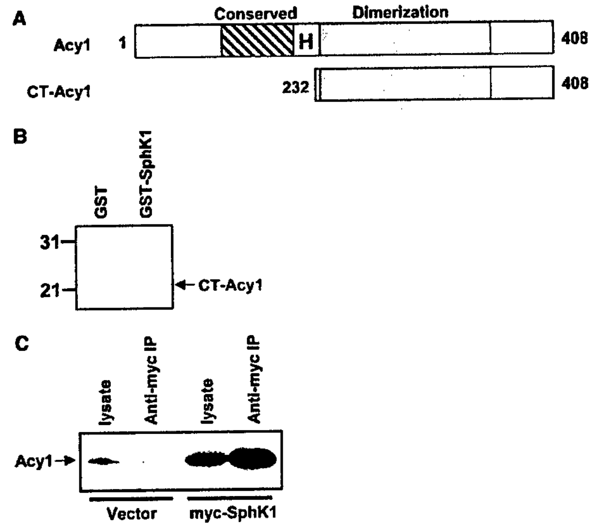

Fig. 1. SphK1 physically interacts with Acy1. (A) Schematic representation of full-length Acy1 (top) and CT-Acy1, the C-terminal fragment pulled out of the two-hybrid screen. Hatched box indicates conserved regions (aa 78–148) amongst Acy1 family members across kingdoms, H indicates the conserved catalytic histidine, and shaded boxes indicate putative dimerization domains. (B) [^{3}H]-labeled CT-Acy1 prepared by in vitro transcription–translation was incubated with either GST or GST-SphK1. Glutathione–Sepharose beads were then added. After overnight incubation at 4 °C, beads were washed and bound proteins resolved by SDS–PAGE and autoradiographed. GST-SphK1 precipitated 22 kDa radiolabeled CT-Acy1. The data are representatives of two independent experiments. (C) HEK 293 cells were co-transfected with V5-Acy1 and either vector or myc-SphK1. Cells were then lysed and immunoprecipitated with anti-myc antibodies followed by protein A/G–Sepharose. The pellets were resolved by SDS–PAGE and immunoblotted with anti-V5. Lysate indicates 1/100 of the total protein immunoprecipitated. Similar results were obtained in two additional experiments.

cytosolic homodimeric metalloenzyme of amino acid salvage [23], catalyzing the hydrolysis of amide-linked Acyl chains of amino acids. It is the major acylase that degrades *N*-acetylcysteine [24], and thus may play a role in the regulation of cellular redox status. Acy1 is abundant in the kidney and brain [24], two tissues with high SphK1 levels [25]. CT-Acy1 is not expected to be active because it lacks conserved residues necessary for binding essential Zn ions and it has a truncated catalytic domain [26] (Fig. 1A).

To examine whether CT-Acy1 interacts physically with SphK1, [^{3}H]-labeled CT-Acy1 was synthesized by in vitro transcription–translation, incubated with either GST or GST-SphK1 [20] and binding was determined using glutathione–Sepharose beads. Sepharose-bound proteins were then resolved by SDS–PAGE and ^{3}H-labeled proteins visualized by autoradiography. CT-Acy1 specifically interacted with GST-SphK1, but not with GST alone (Fig. 1B).

3.2. Acy1 interacts with SphK1 in vivo

To determine if Acy1 interacts with SphK1 when expressed in mammalian cells, HEK 293 cells were co-transfected with Acy1 and SphK1. Lysates were immunoprecipitated with antibodies to SphK1 and the blots probed with antibodies to either CT-Acy1 or Acy1. Both CT-Acy1 (data not shown) and full-length Acy1 co-immunoprecipitated with SphK1 (Fig. 1C). This result, coupled with the GST pull-down results

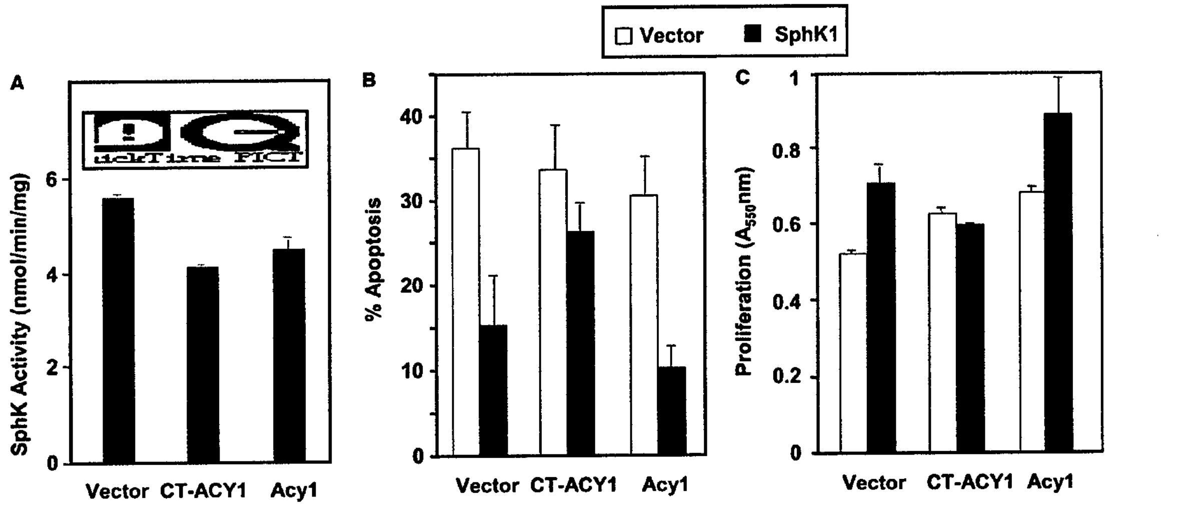

Fig. 2. Effect of Acyl on activity and function of SphK1. (A) SphK1 activity. HEK 293 cells were co-transfected with myc-SphK1 and vector, CT-Acy1, or Acy1. After 48 h, cells were lysed and SphK1 activity measured. Inset shows equal expression of SphK1 as determined by Western blotting with anti-myc. The data are representatives of three independent experiments. (B) Cytoprotective effects of SphK1. NIH 3T3 cells stably transfected with vector (open bars) or SphK1 (filled bars) were transiently transfected with either Acy1, CT-Acy1, or empty vector, together with GFP at a 5:1 ratio, and then serum-starved. After 24 h, cells were fixed and stained with Hoechst. Total GFP-expressing cells and GFP-expressing cells displaying fragmented nuclei indicative of apoptosis were enumerated. Data are means ± S.D. Three independent wells were counted for each treatment, with a minimum of 100 cells scored per well. Data are representatives of two independent experiments. (C) Proliferative effects of SphK1. Cells transfected with the indicated constructs were plated at equal density and allowed to grow for 24 h. Cell proliferation was determined by MTT dye reduction.

and the original two-hybrid data, indicates that SphK1 and Acy1 physically interact in vivo.

3.3. Effects of Acy1 on SphK1 activity and biological functions

We next examined whether the physical interaction with Acy1 affects SphK1 biological functions. Co-transfection of SphK1 with either CT-Acy1 or Acy1 slightly decreased SphK1 activity measured in vitro, without affecting its expression level (Fig. 2A). The best characterized biological responses of SphK1 are suppression of apoptosis and stimulation of cell proliferation and entry into S phase [4,21]. NIH 3T3 cells expressing either vector or SphK1 were co-transfected with CT-Acy1 or Acy1 and effects on apoptosis induced by serum-withdrawal determined by examining chromosomal condensation and fragmentation. Interestingly, in contrast to their inhibitory effects on SphK1 activity, CT-Acy1 reduced while Acy1 potentiated the anti-apoptotic effect of SphK1 (Fig. 2B).

To address the possibility that interaction of Acy1 with SphK1 regulates its mitogenic effect, we also examined the effect of CT-Acy1 or Acy1 on proliferation. In agreement with other studies [27–30], expression of SphK1 increased cell growth as determined by MTT dye reduction assay. Once again, CT-Acy1 had a different effect than full-length Acy1. Whereas CT-Acy1 reduced the growth-promoting effect of SphK1, Acy1 enhanced it (Fig. 2C).

3.4. Acy1 induces redistribution of SphK1

SphK1 is a cytosolic enzyme, while its substrate sphingosine is a lipid found in membranes. Therefore, it is likely that SphK1 activity is regulated in part by its translocation from the cytosol to membranes. Indeed, several previous studies have shown that SphK1 translocates to membranes upon activation [12,15,16,31]. It was therefore of interest to determine whether Acy1 alters the localization of SphK1. First, we examined the localization of both proteins by immunocytochemistry. In agreement with its cytoplasmic expression [32], Acy1 had a diffuse cytosolic localization when expressed in Cos7 cells (Fig. 3A and B). When SphK1 was expressed alone, it also showed a diffuse cytosolic expression pattern (Fig. 3C and D), with dispersed punctate staining as reported previously [27]. However, when Acy1 and SphK1 were co-expressed, although both were still predominantly cytosolic, there was also co-localization in tubular structures (Fig. 3E–G, arrows) and at or near the plasma membrane as indicated by the yellow color in the merged pictures.

To further substantiate that expression of Acy1 induces redistribution of SphK1 to the plasma membrane, we examined their localization by subcellular fractionation. Transfected cells were lysed by freeze-thawing and centrifuged at $100\,000 \times g$. Pellets were then extracted with 1% Triton X-100, generating a soluble fraction and a TI fraction that contains cytoskeleton proteins, focal adhesions, and lipid rafts. As expected from the immunofluorescence data, when expressed alone, both proteins were predominantly localized to the cytosolic fraction (Fig. 4). Interestingly, when co-expressed with Acy1, a portion of SphK1 shifted from the cytosol to the TS fraction (Fig. 4).

4. Conclusions

Our results suggest that Acy1 is a bona fide SphK1-interacting protein that can influence not only its activity but also its cellular localization. Acy1 also potentiated the mitogenic and cytoprotective effects of SphK1 effects. Surprisingly, the CT-Acy1, which also binds SphK1, reduced these effects. Although the physiological significance of these observations is not yet clear, our data suggest that CT-Acy1 may act as a

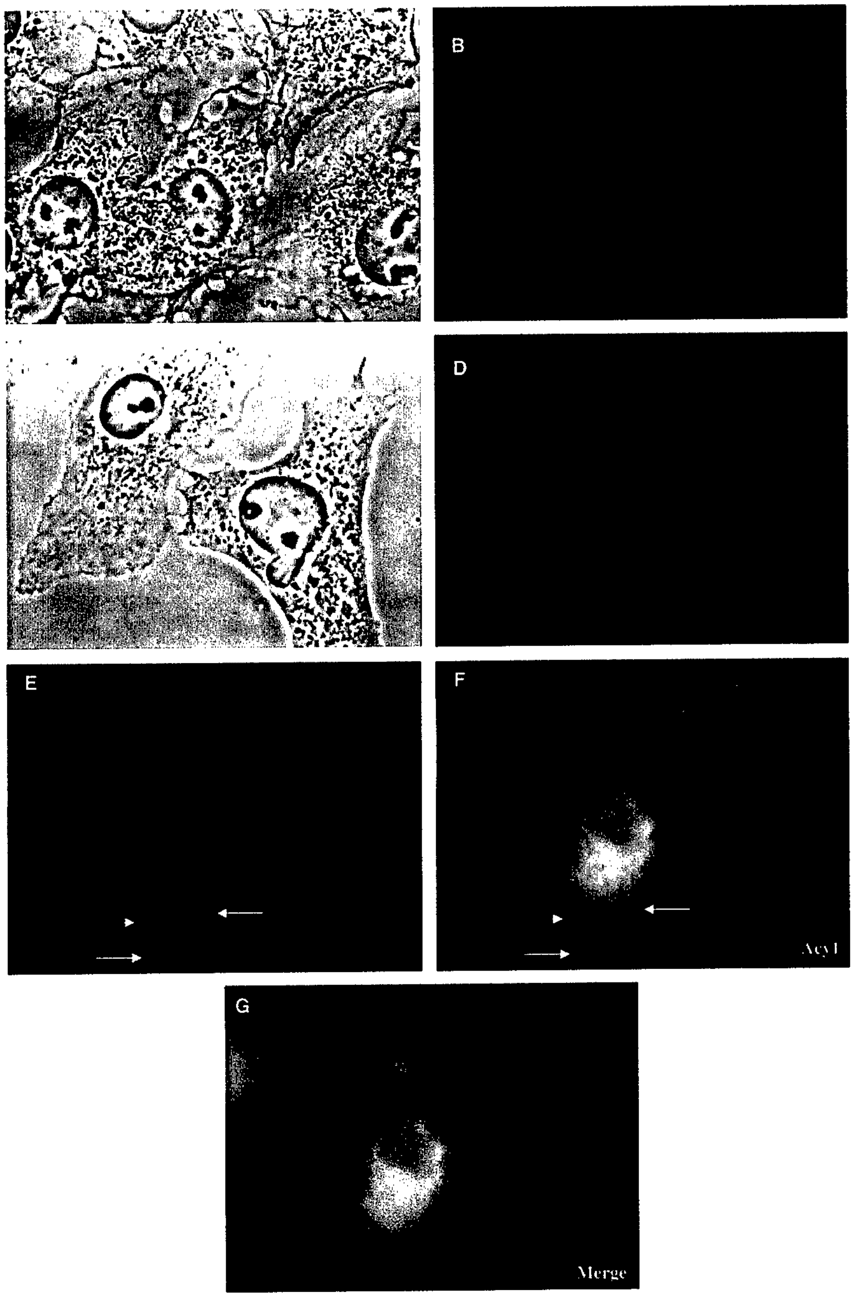

Fig. 3. Acyl alters the intracellular distribution of SphK1. Cos7 cells were transfected with V5-Acyl (A,B) or myc-SphK1 (C,D) or both (E–G) and fixed 48 h later. Cells were then incubated with anti-myc and anti-V5 antibodies and stained with Texas red anti-mouse IgG and FITC anti-rabbit IgG. Phase (A,C) and fluorescent (B,D,E–G) images were obtained with a Nikon TE-200 using a CoolSnap camera driven by MetaMorph software. Panel G shows the superimposed merged pictures, yellow color represents co-localization of the two proteins. Arrows indicate long tubular structures observed only when proteins were co-transfected.

dominant-negative inhibitor of SphK1. We suspect that overexpression of CT-Acyl blocks the ability of SphK1 to interact with endogenous, active Acyl. This would block the pro-growth and anti-apoptotic effects of SphK1 if the aminoacylase activity of Acyl is required for its SphK1 regulatory effects, because CT-Acyl is enzymatically inactive. It is also possible that the N-terminus of Acyl, missing from CT-Acyl, may have binding sites for other proteins required for the SphK1–Acyl complex to inhibit apoptosis and promote cell growth or for its translocation to its site of action.

Because cellular levels of the bioactive sphingolipid mediator S1P are low and tightly regulated, it is not surprising that cells have evolved many mechanisms to control the activity of SphK1, the critical enzyme responsible for formation of S1P, as suggested by the discovery of a plethora of SphK1-interacting proteins [17–20]. Most of them, including Acyl, have in

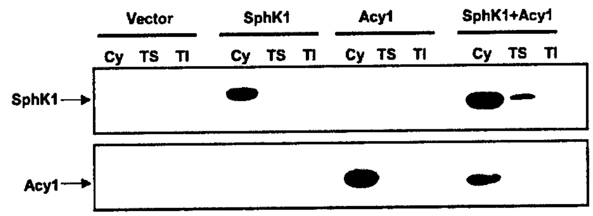

Fig. 4. Acy1 translocates SphK1 from the cytosol to the Triton-soluble (TS) membrane fraction. HEK 293 cells were transfected with SphK1, Acy1, or both. After 48 h, cells were harvested and lysed by freeze-thawing. The lysates were centrifuged at $100\,000 \times g$ to generate cytosol (Cy) and pellet fractions. 1% Triton X-100 was added to the pellet fractions and after centrifugation at $100\,000 \times g$, equal amounts of the Triton X-100-insoluble (TI) and TS fractions were separated on 10% SDS–PAGE, transblotted to nitrocellulose, and probed with antibodies to myc (SphK1) and V5 (Acy1) epitopes.

common the ability to reduce SphK1 enzymatic activity and affect its cellular localization, directing it from a diffuse cytoplasmic expression to specific membranes where S1P production can then be spatially and temporally regulated to influence both intracellular and extracellular signaling pathways.

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